PCT/AU00/00079

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I, ANNA MAIJA MADL, ACTING TEAM LEADER EXAMINATION SUPPORT & SALES hereby certify that annexed is a true copy of the Provisional specification in connection with Application No. PP 8577 for a patent by MEDVET SCIENCE PTY LTD and ST. VINCENT'S INSTITUTE OF MEDICAL RESEARCH filed on 09 February 1999.

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PROVISIONAL SPECIFICATION

Invention Title: CYTOKINE-BINDING DOMAIN

Applicant: MEDVET SCIENCE PTY. LTD. and
ST. VINCENT'S INSTITUTE OF MEDICAL RESEARCH

The invention is described in the following statement:

CYTOKINE-BINDING DOMAIN

The present invention relates to a cytokine binding domain, and to a cytokine-binding antagonist and/or a cytokine-binding agonist. The invention further relates to methods of identifying such compounds and uses of such compounds in therapy, prevention and diagnosis.

BACKGROUND OF THE INVENTION

Heterodimeric cytokine receptors comprise two (or three) subunits which subserve distinct and specialised functions. These include a major ligand-binding subunit (the α subunit) and a signalling subunit (the β or γ subunit). Importantly, the latter is able to recognise several cytokines complexed to the appropriate α chain and transduce their signals. This is exemplified by the common β chain (β_c) of the human granulocyte-macrophage colony-stimulating factor GM-CSF, interleukin-3 (IL-3) and IL-5 receptors, the common IL-2 receptor γ chain (shared by the IL-2, IL-4, IL-7, IL-9 and IL-15 receptors) and gp130 (shared by the IL-6, IL-11, LIF, ciliary neutrophic factor, oncostatin M and cardiotrophin receptors). Significantly, IL-5, IL-3 and GM-CSF, the only three cytokines known to stimulate eosinophil production, can be found concomitantly elevated in lungs affected by allergic inflammation.

The simultaneous antagonism of all three GM-CSF, IL-3 and IL-5 may be desirable or indeed necessary for stimulating eosinophils. For example, eosinophils which are believed to be the major cell type involved in allergy can be maintained in numbers and be stimulated by either IL-3, GM-CSF or IL-5 (Lopez *et al*, 1989). Antagonism of all three cytokines may thus be necessary to inhibit the actions of eosinophils and basophils. Similarly, basophils which are also believed to play an effector role in allergy can be stimulated by either IL-3, GM-CSF or IL-5 (Lopez *et al*, 1990). Antagonism of GM-CSF, IL-3 and IL-5 may be accomplished by the concomitant administration of specific antagonists for each different cytokine. Though feasible, this approach has the disadvantage of having to administer up to three different proteins which is not only inconvenient but which also increases the risk of immunogenicity and other side-effects.

Because all three of these cytokines act through a common receptor subunit (β_c) it may be possible to simultaneously inhibit the action of GM-CSF, IL-3 and IL-5 with a single compound via the (β_c) subunit.

Thus, an antagonist directed against the β_c chain may simultaneously inhibit the function of all three cytokines and may prove a useful therapeutic.

One of the major problems in seeking structural data of the binding site of a communal subunit complexed to cytokines is that, unlike homodimeric receptors or isolated α chains of heterodimeric receptors which can directly bind to cytokines, communal subunits cannot bind to cytokines by themselves. To overcome this problem applicants have developed a monoclonal antibody (Mab) against a region which is important for cytokine high affinity binding within domain 4 of the GM-CSF/IL-3/IL-5 common beta chain ($D4\beta_c$) receptor. This Mab, termed BION-1, inhibited the high affinity binding of GM-CSF, IL-3 and IL-5 to human eosinophils, and inhibited their *in vitro* production and functional activation. BION-1 thus represents the first common antagonist of the GM-CSF, IL-3 and IL-5 receptors and a unique tool with which to explore the cytokine-binding site in the common beta chain.

The molecular basis for the affinity conversion of β_c to each ligand is not fully understood as the ligand-receptor complex had not yet been crystallised and this has prevented the structural definition of their ligand-binding sites. Applicants have now crystallised and determined the structure of the $D4\beta_c$ domain of the GM-CSF/IL-3/IL-5 receptor bound to an antagonist in the form of BION-1.

SUMMARY OF THE INVENTION

The present invention provides a cytokine-binding domain or portion thereof which binds to at least one cytokine and is capable of transducing a cytokine signal through a single cytokine receptor, said domain comprising a portion of the B'-C' loop of domain 4 of a β_c chain ($D4\beta_c$) or analogous structure of a cytokine receptor.

More preferably, the domain comprises a portion of the B'-C' loop of domain 4 and a groove which is defined by the B'-C', F'-G' loops and the N-terminal section of domain 4.

Preferably the binding domain is capable of recognising at least two cytokines that have complexed to an α chain. Preferably these cytokines are selected from the group including, but are not limited to, IL-3, IL-5 and GM-CSF or from IL-4 and IL-13. The cytokines particularly IL-3, IL-5 and GM-CSF may bind via the common β_c chain of the cytokine receptor having firstly been bound by the α chain and forming a cytokine: α chain complex.

In another aspect of the invention there is provided a method of identifying a compound having cytokine agonist or antagonist activity said method including

subjecting a potential cytokine agonist and/or cytokine antagonist compound to a cytokine binding domain or portion thereof wherein said domain binds to at least one cytokine and is capable of transducing a cytokine signal through a single cytokine receptor, said domain comprising a portion of the B'-C' loop of domain 4 of a β_c chain or analogous structure of the cytokine receptor;

and

determining the presence of an agonist or antagonist response from the compound on the activity of a cytokine.

More preferably, the domain comprises a portion of the B'-C' loop of domain 4 and a groove which is defined by the B'-C', F'-G' loops and the N-terminal section of domain 4.

The cytokine binding domain may be a domain of a receptor common to a number of cytokines. Preferably the domain is common to GM-CSF, IL-3 and IL-5 or is common to IL-4 and IL-13.

In a preferred aspect there is provided a method of identifying a GM-CSF, IL-3 and IL-5 agonist or antagonist said method including:

subjecting a potential agonist or antagonist to a GM-CSF, IL-3 and IL-5 binding domain or portion thereof wherein said domain binds to at least one of the cytokines and is capable of transducing a cytokine signal through a single cytokine receptor, said domain comprising a portion of the B'-C' loop of domain 4 of a β_c chain or analogous structure of the cytokine receptor; and

determining the presence of an agonist or antagonist response from the compound on the activity of GM-CSF, IL-3 and IL-5.

More preferably, the domain comprises a portion of the B'-C' loop of domain 4 and a groove which is defined by the B'-C', F'-G' loops and the N-terminal section of domain 4.

In another preferred aspect of the invention there is provided a method of identifying a compound having a cytokine antagonist activity, said method including:

subjecting a potential cytokine antagonist to a cytokine binding domain or portion thereof wherein said domain or portion thereof binds to at least one cytokine and is capable of transducing a cytokine signal through a single cytokine receptor, said domain comprising a portion of the B'-C' loop of domain 4 of a β_c chain or analogous structure of the cytokine receptor; and

identifying a compound that has bound to the cytokine-binding domain wherein said compound has an antagonist response on the activity of the cytokine.

More preferably, the domain comprises a portion of the B'-C'loop of domain 4 and groove which is defined by the B'-C', F'-G' loops and the N-terminal section of domain 4.

In a preferred embodiment, there is provided a method for identifying an antagonist of GM-CSF, IL-3 and IL-5, said method including:

subjecting a potential cytokine antagonist to a cytokine binding domain or potion thereof wherein said domain or portion thereof binds to at least one of the cytokines and is capable of transducing a cytokine signal through a single cytokine receptor, said domain comprising a portion of the B'-C' loop of domain 4 of a β_c chain or analogous structure of the cytokine receptor; and

identifying a compound that has bound to the cytokine-binding domain wherein said compound has an antagonist response on the activity of the cytokine.

More preferably, the domain comprises a portion of the B'-C' loop of domain 4 and a groove which is defined by the B'-C', F'-G' loops and the N-terminal section of domain 4.

In another aspect of the invention there is provided a method of preventing or treating a cytokine - related condition, said method including administering to a subject an effective amount of a compound, agonist or antagonist identified by the methods as described above.

DESCRIPTION OF THE FIGURES

Figure 1 illustrates the structure of D4β_c. **(A)** Structure of the Fab-receptor complex shown in ribbon representation. The light chain is shown in gray, the heavy chain in dark blue and the receptor in gold. The locations of key residues are denoted by ball-and-stick. This picture was produced using MidasPlus. **(B)**,**(C)** Surface representations of the receptor using the program GRASP. The green surfaces indicate the location of hydrophobic patches, H1 and H2 respectively. **(D)** Residues surrounding the cytokine-binding domain.

Figure 2 illustrates mutation of either the B′-C′ loop or Tyr 421 of the F′′-G′ loop disrupts functional activation of the receptor. HEK293T cells were co-transfected with constructs encoding mutant β_c together with IL-3 receptor α chain and JAK-2. After 48 hours post-transfection, cells were stimulated with IL-3 at the concentrations specified for 5 minutes and lysates prepared. Immunoprecipitation was carried out with an anti-β_c Mab, 8E4, and the immunoprecipitated proteins separated on 7.5% SDS-PAGE. Proteins were transferred to nitrocellulose and immunoblotted with anti-phosphotyrosine antibody, PY20. The blot was developed by ECL and then stripped and re-probed with anti-β_c antibody, 1C1.

Figure 3 illustrates a surface representation of the hexameric GM-CSF receptor complex model, looking side-on **(A)** and from above **(B)**. α chain is in red, β chain in yellow, and each GM-CSF monomer in magenta and cyan.

Figure 4 illustrates a stereoview of the interactions between the antibody and D4β_c. The heavy chain is shown in dark blue, the light chain in light blue, the B′-C′ loop in red and the F′′-G′ loop in yellow.

Figure 5 illustrates a stereoview $2F_{obs}$-F_{calc} electron density maps showing key regions of the receptor. The maps were calculated from the final model and contoured at 1σ. **(A)** The B′-C′ loop. **(B)** The extended WSXWS box.

Figure 6 illustrates mutation of either the B-C loop or Y421 in the F-G loop disrupts high affinity GM-CSF and IL-3 binding and combination of both these mutations disrupts functional activation of the receptor. **A.** Cos cells were transfected with constructs encoding either wild type (O) or mutant β_c; ^{365}AAAA^{368} (●), Y421A (▲), Y421F (◆) together with the appropriate receptor α chain and saturation binding studies with radio-iodinated cytokine carried out 48 hours post transfection. Binding analysis was analysed by scatchard transformation and affinities determined using the Biosoft Ebda Ligand programme. **B.** HEK293T cells were co-transfected with constructs encoding mutant β_c together, IL-3 receptor α chain and JAK-2. 48 hours post transfection cells were stimulated with IL-3 at the concentrations specified for 5 minutes and lysates prepared. Immunoprecipitation was carried out with an anti-β_c Mab, 8E4, and the immunoprecipitated proteins separated on 7.5% SDS-PAGE. Proteins were transferred to nitrocellulose and immunoblotted with anti-phophotyrosine antibody, PY20. The blot was developed by ECL and then stripped and re-probed with anti-β_c antibody, ICI.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a cytokine-binding domain or portion thereof which binds to at least one cytokine and is capable of transducing a cytokine signal through a single cytokine receptor, said domain comprising a portion of the B'-C' loop of domain 4 of a β_c chain or analogous structure of a cytokine receptor.

More preferably, the domain comprises a portion of the B'-C' loop of domain 4 and a groove which is defined by the B'-C', F'-G' loops and the N-terminal section of domain 4.

In another embodiment, the domain further includes a Tyrosine residue capable of interaction with an α chain to enhance binding of the cytokine. In a further preferred embodiment, the tyrosine is Tyr 421. The Tyr 421 improves the receptor-receptor interaction or oligomerisation.

Preferably the binding domain is capable of recognising and binding to at least two cytokines that have complexed to an appropriate α chain. Preferably these cytokines are selected from the group including, but are not limited to, IL-

3, IL-5 and GM-CSF or to IL-4 and IL-13 The three cytokines IL-3, IL-5 and GM-CSF may be bound via the common β_c chain of the cytokine receptor having firstly been bound by the α chain and forming a cytokine: α chain complex. Similarly, IL-4 and IL-13 may share a common β_c on the cytokine receptor.

The common β_c chain may derive from any one of the following, GM-CSF, IL-3 and IL-5 receptors, the common IL-2 receptor γ chain (shared by the IL-2, IL-4, IL-7, IL-9 and IL-15 receptors) and gp130 (shared by the IL-6, IL-11, LIF, ciliary neutrophic factor, oncostatin M and cardiotrophin receptors) or from any one of the cytokine superfamily receptors but not limited to the group comprising LIFR, gp130, IL-2Rβ, IL-4R/IL-13R, IL-2Rγ, IL-3Rα, EPOR, TPOR and OBR or be selected from a related (class 1) cytokine receptor structure selected from the group including but not limited to growth hormone receptor (GHR), prolactin receptor (PRLR), erythropoietin receptor (EPOR), G-CSF receptor (G-CSFR) and gp130. The pairwise sequence identities between D4β_c and these receptors, after structure-based alignment, range from 12% (G-CSF) to 27% (gp130). There are only seven residues (Pro 343, Trp 358, Leu 402, Tyr 408, Arg 413, Gly 423, Ser 426) that are strictly conserved across the receptors, all of which appear to play structural roles. A structural superposition indicates that D4β_c is most closely related to PRLR (r.m.s. deviation of 1.4 Å on 86 Ca atoms, 20% sequence identity) followed by GHR (r.m.s. deviation of 1.5 Å on 81 Ca atoms, 23% sequence identity).

Preferably, the common β_c chain is derived from IL-5, IL-3 or GM-CSF receptor. These cytokines IL-3, IL-5 and GM-CSM are known to stimulate eosinophil production and can be found concomitantly elevated in lungs affected by allergic inflammation. Their simultaneous elevation may increase eosinophil numbers, contribute to the overall degree of eosinophil activation, be responsible for the different phases of eosinophil infiltration and determine a localised versus a generalised eosinophil-mediated inflammation. This may be particularly important in the pathology of certain disease such as asthma where the eosinophil plays an effector role.

The common β_c chain of domain 4 ($D4\beta_c$) has been found to have a compact globular shape with overall dimensions of 45 Å x 25 Å x 20 Å (Fig. 1A). The N- and C- termini represent the sites of attachment for the remainder of the extracellular region and the membrane-spanning domain, respectively. The molecule adopts the topology of a fibronectin type III module with two anti-parallel beta-sheets (42% sheet) packing against each other via a multitude of hydrophobic interactions, including two clusters of aromatic residues (Trp 434, Tyr 354 and Tyr 376; Trp 358, Phe 372 and His 370). Sheet A consists of three beta-strands (A′, B′ and E′) and sheet B consists of five strands (C′, D′, F′, F′′ and G′) with the longest strand, C′, almost spanning the length of the molecule. The amino acid sequence motif, WSXWS (where X is any residue), a characteristic feature of many cytokine receptors, is located between the F′′ and G′ strands and adopts a double β-bulge structure (Fig. 1A). Arginine residues from strand F′ interdigitate between the tryptophan residues of the motif to form a ladder of alternating basic and aromatic residues. The ladder is extended in each direction by additional aromatic and basic residues: Tyr 421 - Arg 415 – Trp 425 - Arg 413 - Trp 428 - Arg 411 - Trp 383 – Arg 377 - Trp 409-Arg 407. There is a "side-step" in the ladder at Arg 377 - Trp 409. This ten-rung ladder, measuring 35 Å long with rungs of about 5 Å wide, represents the only significant electropositive patch on the molecule's surface.

Besides the ladder, there are three other significant surface features worth noting. There are two large hydrophobic patches on the surface. The first, H1, is a dense strip of hydrophobic residues located at one edge of the β-sandwich defined by the D′ and E′ strands and measures 27 Å long and 6 Å in width (Fig. 1B). The second, H2, located on the opposite face to the first, forms part of a lip at the end of a pronounced groove on the surface of the molecule (Fig. 1C). The H2 patch is made up of residues Ile 338, Ala 341, Met 361, Tyr 365 and the aliphatic moiety of Lys 362. The groove is located at the N-terminal end of the molecule where one wall is formed by the B′-C′ loop and part of the F′′-G′ loop and the other wall by the N-terminus (residues 338 to 342) (Fig. 1C).

The B'-C' loop of domain 4 or the common β_c chain ($D4\beta_c$) of the cytokine receptor or part thereof is involved in the cytokine binding. It adopts a regular structure in $D4\beta_c$ having residues 360 to 363 forming a type VIII β-turn and residues 365 to 368 forming a type I β- turn. Preferably the portion of a the B'-C' loop of the domain includes Tyr 365, Ile 368 and His 367. GCSFR, GHR, and PRLR have an aromatic residue equivalent to Tyr 365, whereas there is no corresponding residue to His 367.

In the interaction of a cytokine with B'-C' loop it is further preferable that the residues Tyr 365, His 367 and Ile 368 are involved in the interaction of the cytokine and the receptor. Ideally, the Tyr 365, His 367 and Ile 368 form a cytokine binding triad that converges to form a pivot point to which all three cytokines (GM-CSF, IL-3 and IL-5) may bind via essential glutamate residues (Glu 21 of GM-CSF, Glu 22 of IL-3 and Glu13 of IL-5).

The F'-G' loop adopts a type IV β turn at its tip in $D4\beta_c$ and the most significant features in this region are Arg 418 and Tyr 421, both of which projects out of solution (Fig. 1A). Accordingly, when the cytokine interacts with the β_c chain, the Tyr 421 interacts with the α-chain to enhance receptor-receptor interaction or oligermerisation.

More preferably, the domain comprises a portion of the B'-C' loop of domain 4 and a groove which is defined by the B'-C', F'-G' loops and the N-terminal section of domain 4.

In a further preferred embodiment the binding domain or portion thereof capable of binding the cytokine may be defined by an area bordered by any of the following residues Lys 362, Tyr 365, His 367, Ile 368, Arg 418, Gly 420, Asn 422, Thr 416, Ile 338, Gln 339, Met 340 and Met 361. A majority of these residues are in the B'-C' loop and hence constitute a portion of the B'-C' loop capable of transducing a cytokine signal. The binding domain may be described as a "groove" comprising a concave surface formed largely, but not exclusively by hydrophobic residues, preferably of those listed above.

The hydrophobic surface patches, H1 and H2, of $D4\beta_c$ (Figs. 1B, 1C) have corresponding features in most of the other receptors. With the exception of gp130, all the receptors possess significant hydrophobic patches equivalent

to the location of H1 (centered about the D′-E′ strand connection), although the degree and extent of hydrophobicity varies greatly. The equivalent region to H2 is conserved in all but gp130. By analogy with the other receptors, the H2 patch of D4β_c might interact with the A-B loop from domain 3 of the intact receptor.

In another aspect of the invention there is provided a method of identifying a compound having cytokine agonist or antagonist activity said method including

subjecting a potential cytokine agonist and/or cytokine antagonist compound to a cytokine binding domain or portion thereof wherein said domain binds to at least one cytokine and is capable of transducing a cytokine signal through a single cytokine receptor, said domain comprising a portion of the B'-C' loop of domain 4 of a β_c chain or analogous structure of the cytokine receptor; and

determining the presence of an agonist or antagonist response from the compound on the activity of a cytokine.

More preferably, the domain comprises a portion of the B'-C' loop of domain 4 and a groove which is defined by the B'-C', F'-G' loops and the N-terminal section of domain 4.

The common β_c chain may be common to IL-3, IL-5 or GM-CSF or it may be common to IL-4 and IL-13.

The agonist or antagonist response may be measured by the ability of the compound to activate or inhibit a cytokine response. This can be measured by cellular activities associated with any of the cytokines. For instance, with the cytokines IL-5, GM-CSF and IL-3 stimulation of eosinophil adherence, priming for degranulation and cytotoxicity, and propagation of viability may be measured in the presence or absence of the agonists or antagonists binding to the cytokine binding domain or a portion thereof as hereinbefore described.

In a preferred aspect there is provided a method of identifying a GM-CSF, IL-3 and IL-5 agonist or antagonist said method including:

subjecting a potential agonist or antagonist to a GM-CSF, IL-3 and IL-5 binding domain or portion thereof wherein said domain binds to at least one of

the cytokines and is capable of transducing a cytokine signal through a single cytokine receptor, said domain comprising a portion of the B'-C' loop of domain 4 of a β_c chain or analogous structure of the cytokine receptor; and

determining the presence of an agonist or antagonist response from the compound on the activity of GM-CSF, IL-3 and IL-5.

More preferably, the domain comprises a portion of the B'-C' loop of domain 4 and a groove which is defined by the B'-C', F'-G' loops and the N-terminal section of domain 4.

In another preferred aspect of the invention there is provided a method of identifying a compound having a cytokine antagonist activity, said method including:

subjecting a potential cytokine antagonist to a cytokine binding domain or portion thereof wherein said domain or portion thereof wherein said domain binds to at least one cytokine and is capable of transducing a cytokine signal through a single cytokine receptor, said domain comprising a portion of the B'-C' loop of domain 4 of a β_c chain or analogous structure of the cytokine receptor; and

identifying a compound that has bound to the cytokine-binding domain wherein said compound has an antagonist response on the activity of the cytokine.

More preferably, the domain comprises a portion of the B'-C' loop of domain 4 and a groove which is defined by the B'-C', F'-G' loops and the N-terminal section of domain 4.

In a preferred embodiment, there is provided a method for identifying an antagonist of GM-CSF, IL-3 and IL-5, said method including:

subjecting a potential cytokine antagonist to a cytokine binding domain or portion thereof wherein said domain or portion thereof binds to at least one of the cytokines and is capable of transducing a cytokine signal through a single cytokine receptor, said domain comprising a portion of the B'-C' loop of domain 4 of a β_c chain or analogous structure of the cytokine receptor; and

identifying a compound that has bound to the cytokine-binding domain wherein said compound has an antagonist response on the activity of the cytokine.

More preferably, the domain comprises a portion of the B'-C' loop of domain 4 and a groove which is defined by the B'-C', F'-G' loops and the N-terminal section of domain 4.

The antagonist preferably inhibits the binding of cytokines preferably IL-3, IL-5 and GM-CSF or IL-4 and IL-13 to the β_c chain via a portion of the B'-C' loop. Preferably that portion is identified by the "groove" or part thereof as described above. The cytokines may bind the common β_c chain and more particularly to a portion of the B'-C' loop.

The methods identified above will allow the identification and design of agonists and antagonist of cytokines that can act through the portion of the β_c chain of a cytokine receptor or an analogous common chain in other receptors. Preferably the β_c chain is common to GM-CSF, IL-3 and IL-5 or to IL-4 and IL-13 or is an analogous chain common chain in other receptors.

A crystalline form of the cytokine binding domain is also provided in the present invention and will allow for structure based design of drugs or targeted selection by phage display. Potentional agonists and antagonists may be identified by screening for "groove binders" (compounds that may bind in the groove). These may be determined by considering wild type versus mutant domain 4 molecules. Mutants may be generated using mutations to alanines in the floor of the groove. Mutations may be directed to any of the residues selected from the group including Gln 340, Ile 338 and Met 361 to make the mutants.

Further uses of the crystalline structure allows for affinity maturation using designed mutations such as those in the floor of the groove and including mutations at Gln 340, Ile 338 and/or Met 361. Because applicants have deduced the crystalline structure antibody and antigen structures are further understood by the present description and development of BION-1 mimetics either peptide or non-peptide is included in the scope of this application.

In another aspect there is provided a compound, agonist or antagonist identified by the methods described above. The compound agonist or antagonist may be an antibody or fragment thereof directed to the cytokine binding domain. The antibody may be monoclonal or polyclonal or an active portion thereof.

Methods of making such antibodies will be familiar to those skilled in the art and will be understood to further include the steps of inoculating an animal with a peptide molecule having the cytokine binding domain or a portion thereof as described above, fusing antibody producing cells with a myeloma cell line and screening for a cell line that produces an antibody reactive with the cytokine-binding domain or portion thereof, and harvesting antibodies from the cell line, testing for inhibition of high affinity binding and testing for inhibition or excitation of function. This may further include making small fragments of antibodies produced by the said cell line capable of binding the cytokine binding domain or portion thereof. The cell line may conveniently be a mouse cell line and the method may include the further step of "humanising" the said antibody fragments by replacing mouse sequences with human sequences in the non-binding regions. Humanizing may be conducted by any methods known to the skilled addressee.

The antibody fragment may be a larger portion such as Fab fragments or much small fragments of the variable region. These fragments may be used as separate molecules or alternatively may form part of a recombinant molecule which is then used for therapeutic purposes. Thus for example the monoclonal antibody may be "humanised" by recombining nucleic acid encoding the variable region of the monoclonal antibody with nucleic acid encoding non-variable regions of human origin in an appropriate expression vector.

The agonist and antagonist compounds of the present invention are not limited to antibodies reactive to the cytokine-binding domain or any portions thereof and which compete with the binding with cytokines. Other compounds including small molecules or synthetic or natural chemical compounds capable of competing with the binding of a cytokine to the cytokine-binding domain or any portion thereof are also included in the present invention.

In another aspect of the invention there is provided a method of preventing or treating a cytokine - related condition, said method including administering to a subject an effective amount of a compound, agonist or antagonist as described above.

The compound, agonist or antagonist may be used singularly or in combination with other therapeutic agents such as corticosteroids.

The cytokine related condition may be a condition associated with any one of the group including GM-CSF, IL-3 and IL-5 or IL-4 and IL-13 or a condition which requires the binding of the cytokines to a common β_c chain

The examples below recite the use of antibodies to the cytokine binding domain as antagonists. However other compounds capable of inhibiting the binding of cytokine will be equally applicable.

The antagonist effect preferably leads to blocking of at least one function of any one of the cytokines which may be bound to a common β_c chain. One of the benefits that is proposed to be derived from these antagonists is their use in modifying cells stimulated by one of the cytokines, and more in one specific form modifying the activity of the cytokines is proposed to impact greatly on cellular functions including eosinophil function. Therefore preferably the activity leads to inhibition of stimulation of effector cell activation and where the antibody or fragment thereof is to be used for treatment of asthma (for example), it leads most preferably to inhibition of IL-5, IL-3 and GM-CSF or IL-4 and IL-13 mediated eosinophil activation. It will be understood however that cells other than eosinophils are also the effectors of adverse conditions in humans and animals as a result of stimulation by these cytokines and inhibition of such stimulation is also contemplated by this invention. These include cells that express either one or all of GM-CSF, IL-3 and IL-5 receptors, or the IL-4 and IL-13 receptors the stimulation of which leads to pathology. Examples of these are leukaemic cells, endothelial cells, breast cancer cells, prostate cancer cells, small cell lung carcinoma cells, colon cancer cells, macrophages in chronic inflammation such as rheumatoid arthritis and dendritic cells for immunosuppression.

A number of different facets of eosinophil function might be modified so that in one form IL-5, IL-3 and GM-CSF or IL-4 and IL-13 mediated eosinophil survival is inhibited or blocked. In a further form IL-5, IL-3 and GM-CSF, or IL-4 and IL-13 mediated eosinophil activation is inhibited or blocked.

The treatment may be aimed at being preventative by reducing the risk of contracting the condition, or the treatment may be used to alleviate or obviate the condition. The administration of the therapeutic agent can be any pharmaceutically acceptable form and in a suitable carrier.

It is thought that the construction of compounds that bind a portion of the B'-C' loop of β_c will be therapeutically useful for intervention in conditions where IL-3, GM-CSF and IL-5 or IL-4 and IL-13 play a pathogenic role, mainly allergy, asthma, leukaemia, lymphoma and inflammation including arthritis.

Similarly for other cytokine receptors it is thought that antagonists or agonists will be therapeutically useful.

Since gp130 is functionally analogous to β_c in the GM-CSF/IL-3/IL-5 receptor system, in that it is a common binding subunit and signal transducer for the IL-6, oncostatin M (OSM), ciliary neurotrophic factor (CNTF), leukaemia inhibitory factor (LIF) and IL-11, it is suggested that targeting/blocking of this cytokine binding domain will lead to antagonism of the IL-6, LIF, OSM CNTF and IL-11. Antagonism of this receptor system will be useful in inflammation, leukaemia and lymphoma. Antagonist of IL-2Rβ/γ may be useful as immunosuppressants. Antagonists of LIFR may be useful for the prevention of implantation of embryos in uteri. Antagonist of IL-4/IL-13 will inhibit IgE production and may be useful in treating asthma and allergies. [M. Willis-Karp et al (1998)].

Antagonist of IL-3 may be useful in treating allergy and follicular B cell lymphoma. Antagonists of IL-4 may inhibit IgE production, and be useful for treatment of asthma and allergy. Antagonists of IL-6R may be useful as an anti-inflammatory and may be used to inhibit myeloma growth. Antagonists against IL-7 may be usefúl as an immunosuppressant. Antagonists of the leptin receptor (OBR) may be useful in the treatment of cachexia, weight loss in conditions such as AIDS, cancer and parasitic diseases.

Agonists agents that bind to β_c via the B'-C' loop as described above may be used to stimulate hemopoesis, and to boost immune response against microorganisms and parasites. Agonist agents that bind to LIFR may be useful in the suppression of embryonic stem cell differentiation. Agonists agents that bind to IL-2Rβ may be used in immunostimulation. Agonists agents that bind to IL-4R/IL-13 may have anti-tumour activity.

Agonists agents that bind to specific subunits IL-3R may be use in the *in vivo* and *ex vivo* expansion of early hemopoietic cells. Agonists agents that bind to IL-4R may have useful anti-tumour activity. Agonists agents that bind to IL-7R may have useful anti-tumour immunity. Agonists agents that bind IL-11 may prove a useful adjunct to cancer therapy. Agonists agents that bind to EPOR may be used to correct anaemia of chronic renal failure, of chronic inflammatory diseases and of malignant diseases. Agonists agents tat bind to TPOR, may be useful for correcting thrombocytopenia (such as may be associated with chronic inflammatory diseases, malignancies, chemo- and radio- therapy).

Examples of useful agonists are those for erythropoietin and thrombopoietin to elevate erythrocyte and platelet numbers in blood following blood cell loss, chemotherapy, radiotherapy, immunosuppression or bone marrow transplantation. Agonists of OBR may be used to induce weight loss, and in particular for obesity which is considered to be a contributing factor of hypertension, coronary heart disease and noninsulin-dependent diabetes mellitus. The molecules whether agonist or antagonist can be isolated on the basis of their ability to interact with the cytokine binding domain as described above.

The present invention will now be more fully described with reference to the following examples. It should be understood, however, that the description following is illustrative only and should not be taken in any way as a restriction on the generality of the invention described above.

EXAMPLES

Example 1 - <u>Crystallised BION-1-D4β_c complex.</u>

The crystals belong to space group $P4_12_12$ with cell dimensions $a = b$ = 77.6 Å, c = 294.9 Å. A native data set was collected from a single flash-frozen crystal in-house on a MARResearch imaging plate area detector with Cu*Ka* X-rays generated by a Rigaku RU-200 rotating anode generator. The cryoprotectant used was 15% MPD. The diffraction data were processed and analysed using DENZO and SCALEPACK [Z. Otwinowski and W. Minor, (1997)] and programs in the CCP4 suite (Daresbury Laboratory, UK). The processing resulted in a 91% complete data set to 3.3 Å resolution consisting of 13,143 unique reflections. The overall R_{sym} was 12.4% and the multiplicity was 2.5.

The crystal structure was solved straightforwardly by molecular replacement using AMoRe [J. Navaza, (1994)]. The search probe was a mouse Fab fragment with PDB identifier 1YEC [J.B. Charbonnier, et al., (1997)]. Rigid body refinement of the initial solution yielded readily interpretable density for D4β_c. The model of the complex was built with the help of skeletonized maps using the program O [T.A. Jones, J.-Y. et al (1991)] and refined using the maximum likelihood target in the program package CNS [A.T. Brunger et al., (1998)]. In the latter stages a bulk solvent correction and grouped B-factors were applied. The final model yielded a R_{factor} of 23.7% (R_{free} of 29.7%). The quality of the final map was good (Fig. 5) with no breaks in the main-chain connectivity. The final model comprises residues 338 to 438 for D4β_c and all residues for the Fab fragment. The r.m.s. deviations from ideality are 0.008 Å and 1.84° for bond lengths and angles respectively, 0.83° for impropers and 29.5° for dihedrals. The correctness of the tracing is supported by 3D-1D scores that never fall below 0.2 [R. Lüthy, et al (1992)]. 83% of the residues fall in the most favoured region of the Ramachandran plot and none fall in the disallowed regions [R.A. Laskowski, et al (1993)]. Coordinates have been deposited in the Brookhaven Protein Data Bank (accession code 1WJM).

Example 2 - <u>Model of complex between α chain, domains 3 and 4 of β_c and GM-CSF, IL-5 and IL-3.</u>

In order to understand why the β_c chain recognises all three cytokines; applicants have modelled the complex between α chain, domains' 3 and 4 of β_c and each cytokine.

The modeling proceeded as follows: (i) A structure-based sequence alignment of the cytokine-binding homology regions (CHRs) from all known class 1 cytokine receptor structures was performed. The amino acid sequences of the GM-CSF α chain CHR and of domain 3 of β_c were then added and manually aligned. The CHR of the α chain and domain 3 of β_c were built by homology to the GHR crystal structure using the multiple sequence alignment as a guide. Loop regions were constructed from peptide fragment data bases and the models subjected to energy minimisation. The stereochemical quality of each model was judged excellent by the program PROCHECK [R.A. Laskowski, et al (1993)] and their correctness supported by 3D-1D scores that never fell below zero [R. Lüthy, et al (1992)]. (ii) The D4β_c structure was superimposed on the corresponding domain of GHR. (iii) Domain 3 was connected to domain 4 based on the L-shaped orientation seen in the other class 1 cytokine receptors. The positioning was supported by the lack of steric overlap with BION-1 which also recognises a fragment of β_c which includes domain's 3 and 4, the short 2 residue linker between the domains and the finding that the gp130 structure, which has no ligand bound, also has the domains in the same orientation. (iv) GM-CSF [K. Diederichs, et al (1991)] was docked onto its corresponding α chain manually using the GHR complex structure as a starting point and then optimising interactions using all the available mutagenesis data. (v) The cytokine-α chain complex was docked onto the modelled β chain by superimposing the GM-CSF component onto the hormone of the GHR complex (using a site 2 orientation in agreement with mutagenesis data. (vi) Small, rigid body adjustments were made manually on the cytokine-α chain complex, with respect to D4β_c in order to optimise contact between cytokine and beta chain. (vi) The other cytokines, IL-3 [Y. Feng, et al (1996)] and IL-5 [M.V. Milburn et al., *Nature* **363**, 172 (1993)], were superimposed onto GM-CSF of the modelled complex. (vii) The hexameric complex, consisting of 2 α subunits, 2 β subunits and 2 cytokine monomers,

was constructed from the trimeric α subunit/β subunit/cytokine model by assuming a proper twofold exists between the two trimers. (Although deviations from a precise twofold relationship are quite possible, large deviations are not envisaged since all subunits have transmembrane regions that must stay imbedded in the membrane on complex formation). The modeling, together with biochemical data yielded two solutions in which the second trimer was located either clockwise or anticlockwise with respect to the first trimer when viewed down onto the membrane surface (Fig. 3). However, only one solution (Fig. 3) explained the importance of Tyr 421. Intriguingly, each monomer of the GM-CSF crystallographic dimer [Walter, M.R. et al (1992)] and of the IL-5 covalent dimer [M.V. Milburn et al (1993)] bind to each α chain in the preferred complex model.

The resultant model reveals the following: (i) The membrane proximal domain of the α chain homology model possesses an elongated hydrophobic surface patch of dimensions 15 Å by 8 Å. Major contributors to the patch are Val 224, Val 226, Cys 228, Ile 313, Phe 315 and Gly 316 (GM-CSF α chain numbering). Most of these residues are conserved in α chains from different receptors and from different species (data not shown). (ii) The hydrophobic patch of the α chain is close enough to the H1 patch of D4β_c to envisage an interaction between the two; however, the interaction area is likely small, consistent with data showing that there is little or no dimer formation observed in the absence of cytokine (Woodcock, J.M. et al (1997)). The corresponding H1 patch of GHR is also involved in subunit contacts (DeVos, A.M. et al (1992)). There are numerous interactions between each cytokine and the B′-C′ loop. Significantly, the side-chains of Tyr 365, His 367, and Ile 368 form a cytokine-binding triad that converges closely at their tips to form a pivot point to which all three cytokines bind via the essential glutamate (Glu 21 of GM-CSF, Glu 22 of IL-3 and Glu 13 of IL-5) (Hercus, T.R. et al. (1994)). These observations are consistent with mutagenesis data that show Tyr 365, His 367 and Ile 368 are key GM-CSF binding determinants (Lock, P. et al. (1994)). Surprisingly, Tyr 421, the sole residue in the F′-G′ loop implicated in high affinity binding (Woodcock, J.M. et al (1996)) is orientated away from the

cytokine-binding site in the crystal structure (Fig. 1A). Biochemical data also shows that, whilst a Tyr 421 Phe mutation has a significant effect on the phosphorylation of the cytoplasmic domain, mutations in the B'-C' loop have a nominal effect (Fig. 2). An explanation for the critical role of this residue comes from the crystal structure and observations that the α,β heterodimer exists as a higher order complex (Lia, F. et al. (1996), Stomski, F.C. et al (1998)). A heterohexameric complex of two α chains, two β chains and two cytokines has been proposed based on the location of disulfide bridges in the GM-CSF receptor complex (Stomski, F.C. et al. (1998)) and by analogy to the IL-6 receptor system which has been shown to form a similar hexameric complex (Ward, L.D. et al. (1994)). We have modelled the hexameric complex and find that Tyr 421 is in an ideal position to interact with the second α chain of the complex (Fig. 3).

The monoclonal antibody antagonist, BION-1, forms extensive and intimate interactions with the receptor domain (Fig. 4). The total surface area buried on complex formation is 1,500 Å^2, which is in the range reported for other antibody-protein antigen complexes (Davies, D.R. and Cohen G.H. (1996)). In total, there are 2 salt bridges, 8 hydrogen-bonding interactions and 86 van der Waals (vdw) interactions.

The contact surface comprises 15 residues from BION-1 with 9 residues from V_H and 6 residues from V_L. The majority of contacts are roughly shared between four of the CDRs: CDR L1 (1 hydrogen bonds and 24 vdw contacts); CDR L3 (1 salt bridge, 3 hydrogen bonds and 20 vdw contacts); CDR H1 (1 salt bridge, 3 hydrogen bonds and 18 vdw contacts); CDR H3 (1 hydrogen bonds and 15 vdw contacts). In addition, CDR H2 provides a number of contacts (9 vdw contacts) but CDR L2 makes no contacts with the receptor domain. Modeling suggests that CDR L2 interacts with domain 3 of the intact b chain. In total, 6 residues from the B′-C′ loop (between residues 362 and 368) and 4 residues from the F′-G′ loop (between residues 416 and 422) are involved in antibody interactions. The B′-C′ loop interacts with CDRs H1, H2, H3, L1 and L3 whereas the F′-G′ loop interacts only with CDRs H3 and L1. The specific polar interactions between the B′-C′ loop and the antibody are as follows: Lys

362 forms a salt bridge to Asp 94L, Glu 366 forms a salt bridge to Lys 35H, and the following residues form potential hydrogen bonding interactions: Arg 364 and Tyr 33H, Arg 364 (main-chain) and Tyr 33H, Tyr 365 and Glu 93L (main-chain), Tyr 365 and Asp 94L, Glu 366 and Tyr 33H (main-chain), His 367 and Asn 91L (main-chain). There are two potential hydrogen-bonding interactions involving the F′-G′: Thr 416 and Tyr 28L, Arg 418 and Gly 96aH (main-chain). There is one small cavity of 9.9 $Å^3$ in the antibody-antigen interface. The cavity is lined by residues Tyr 365, His 367 and Ile 368 of the receptor and Val 27, Tyr 28, Phe 32 and Asn 92 of the antibody light chain.

The B′-C′ loop of D4β_c is nestled in the shallow antigen-binding groove between the V_H and V_L domains whereas the F′-G′ loop forms a more peripheral interaction with CDR L1 of BION-1. Interactions from the B′-C′ loop account for 75% of the total interactions of D4β_c with BION-1. Of particular note are numerous aromatic interactions involving aromatic residues from BION-1 and Tyr 365 and His 367 of the receptor (Fig. 4). These types of interactions are a common feature at antibody combining sites.

The epitope of D4β_c recognised by BION-1 largely overlaps the surface that interacts with the cytokines. Furthermore, BION-1 inhibited the GM-CSF/IL-3/IL-5-induced proliferation of eosinophils *in vitro*, highlighting the feasibility of single molecule antagonists of several cytokines. This multi-hit approach may prove useful in allergic inflammation and cancer where more than one cytokine is frequently associated with these diseases. The structure presented here provides a number of possibilities for the design of novel therapeutics: (i) The affinity of BION-1 for intact β chain is 50 nM in contrast to high affinity cytokine-binding of 0.1 nM. The structure provides the opportunity of engineering a higher affinity antibody or corresponding small molecule mimetic, such as a cyclized, mutated version of a major contributing CDR from BION-1. (ii) The presence of the groove at the cytokine-binding interface is an appealing site for the design of small molecule antagonists. (iii) The location of Tyr 421 at a critical subunit interface provides another distinct target for structure-based inhibitor design.

Finally it is to be understood that various other modifications and/or alterations may be made without departing from the spirit of the present invention as outlined herein.

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DATED: 8 February, 1999

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FIGURE 1A

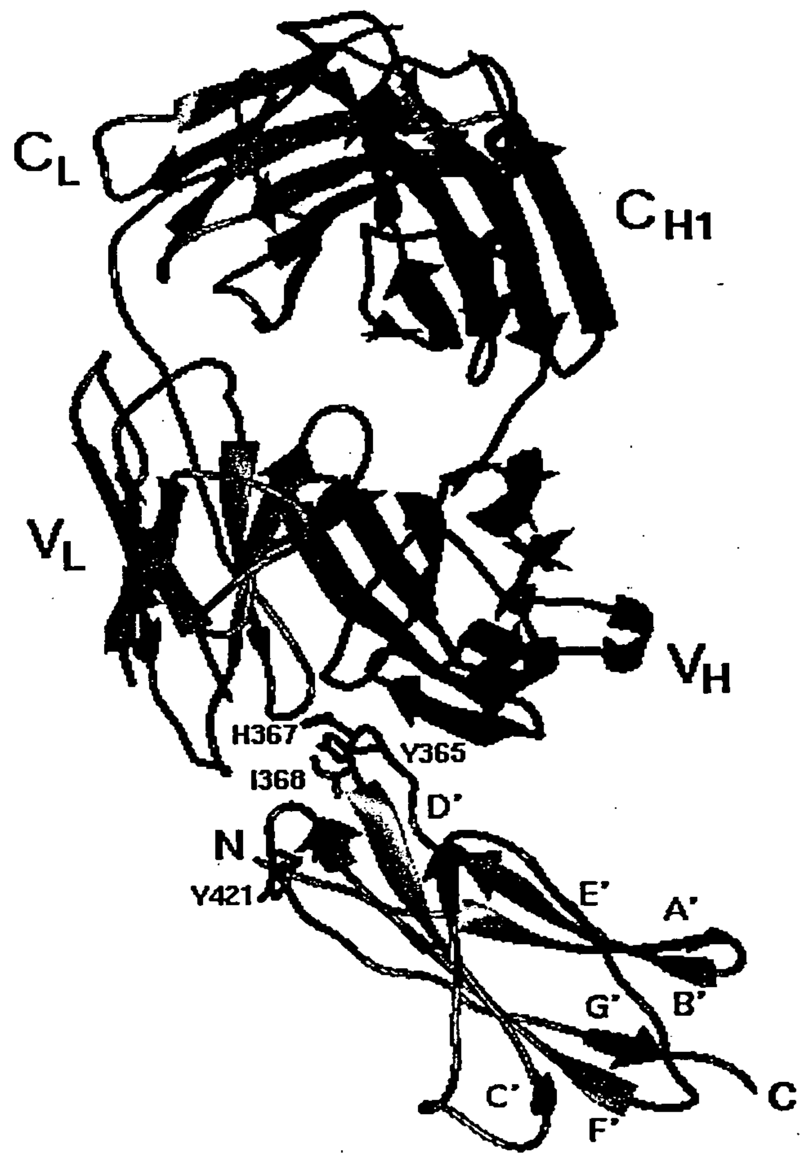


FIGURE 1B

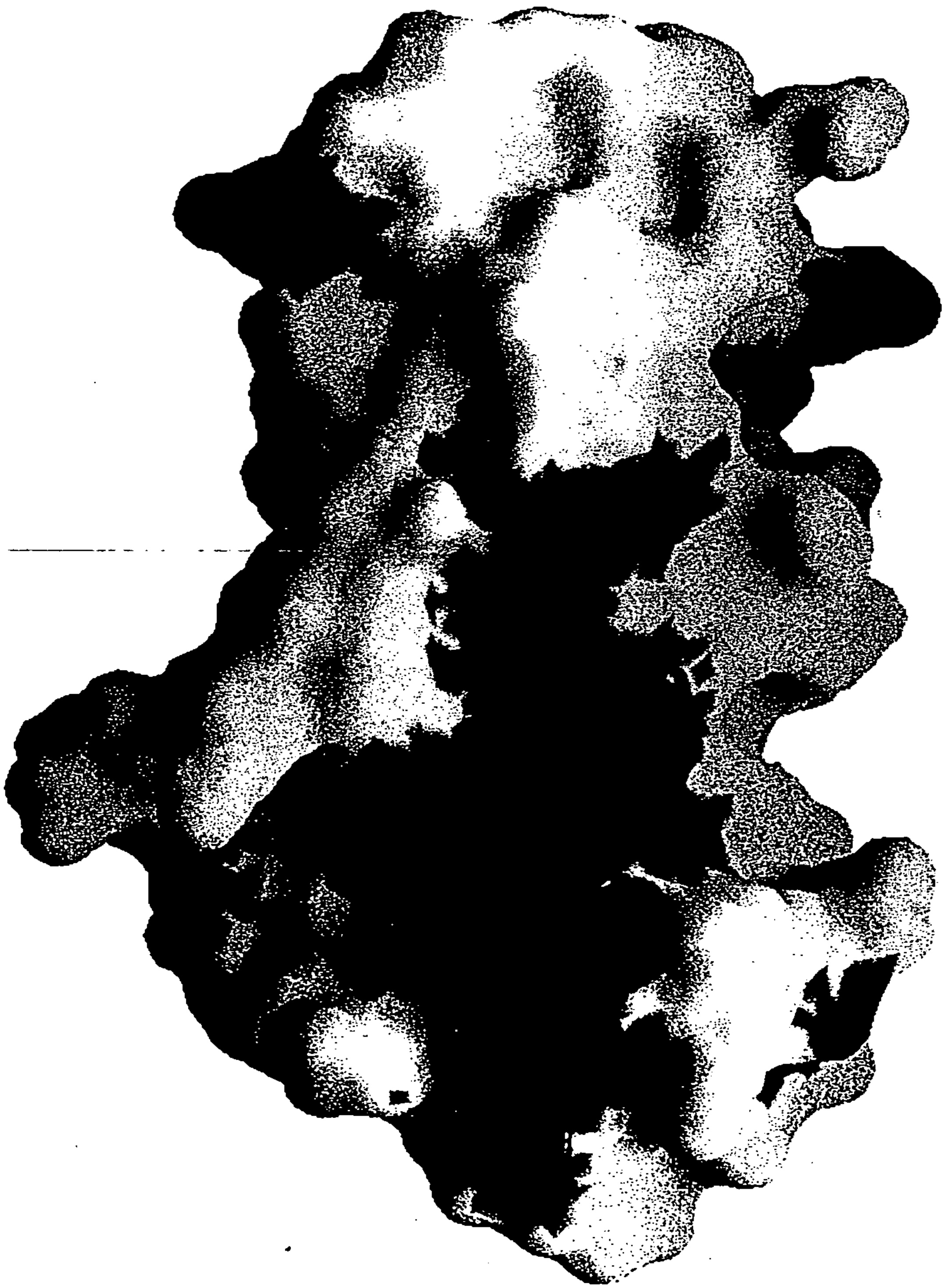


FIGURE 1C

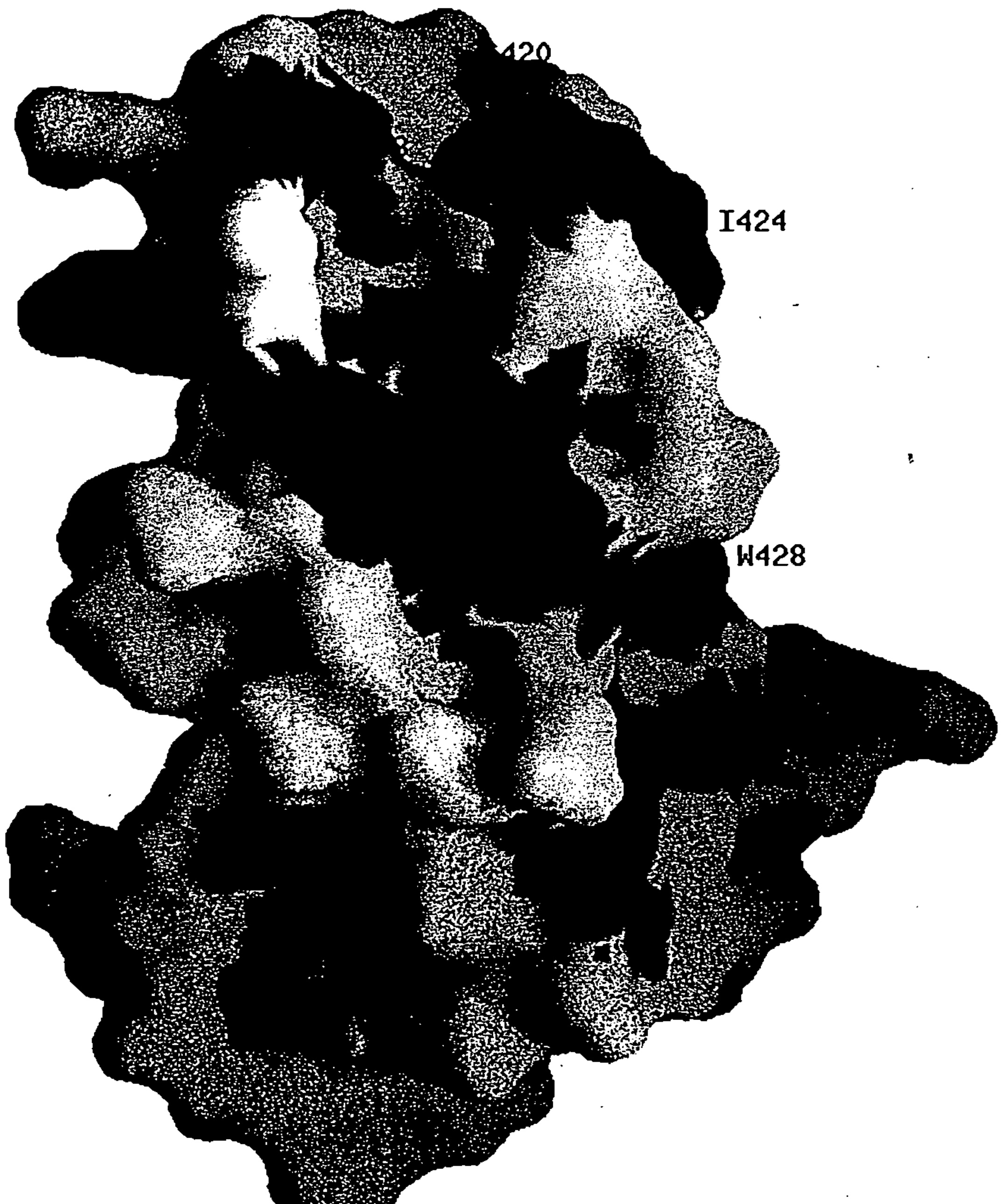


FIGURE 1D

Fig. 1c

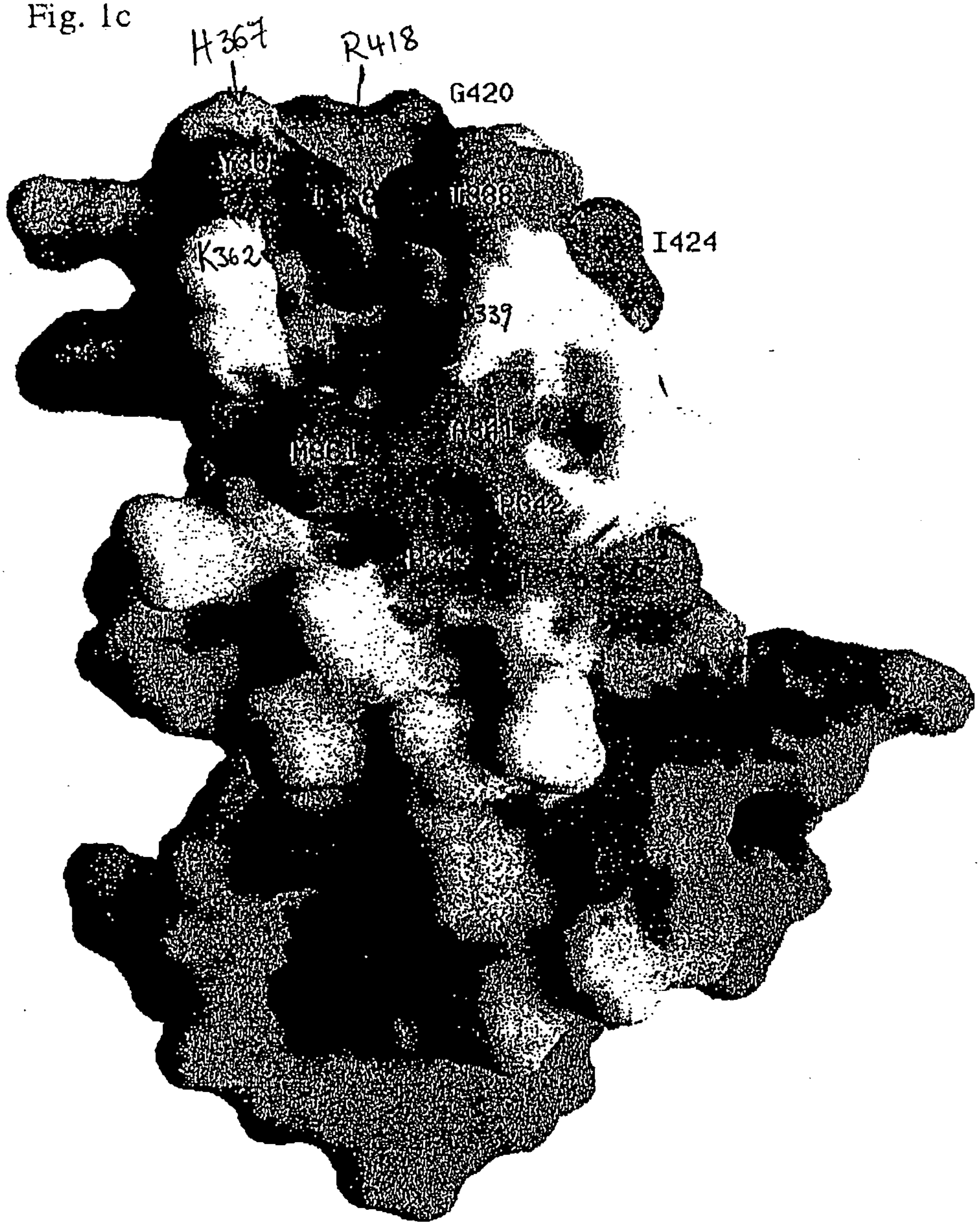


FIGURE 2

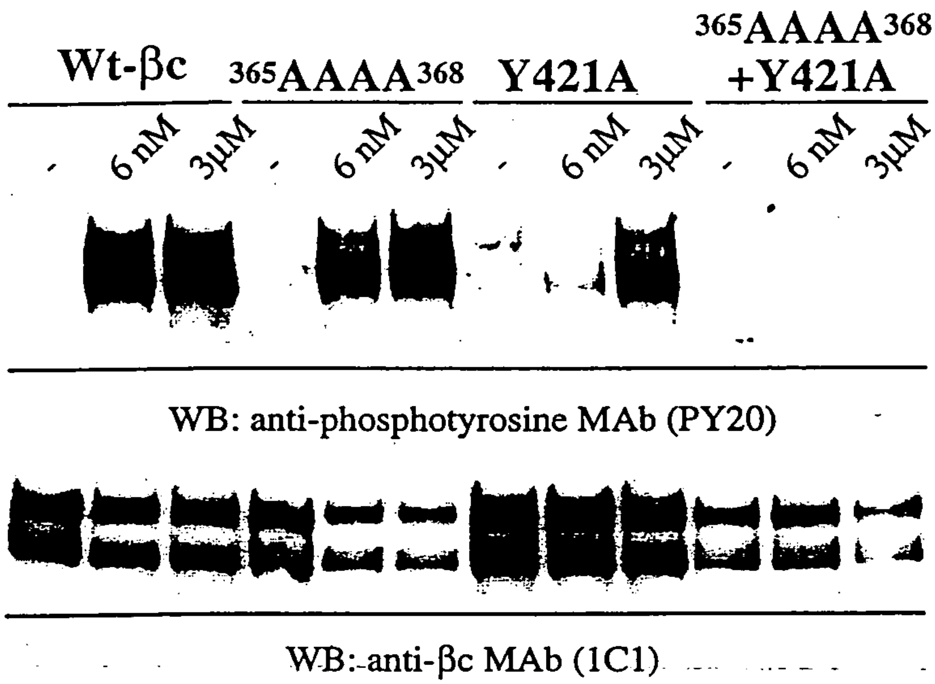

Wt-βc
365AAAA368
Y421A
365AAAA368 +Y421A
- 6 nM 3μM
- 6 nM 3μM
- 6 nM 3μM
- 6 nM 3μM
WB: anti-phosphotyrosine MAb (PY20)
WB: anti-βc MAb (1C1)

FIGURE 3

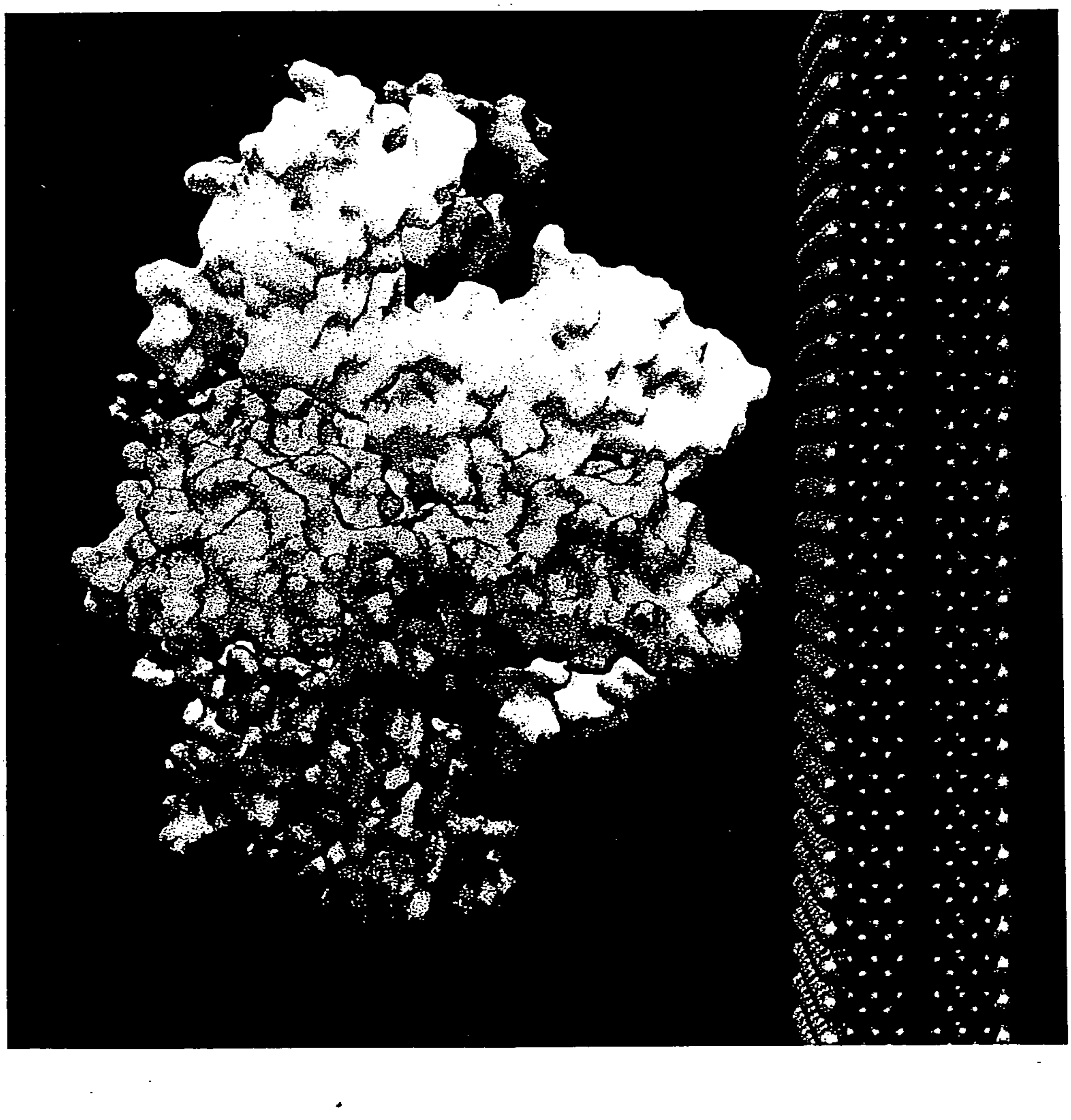


FIGURE 3B

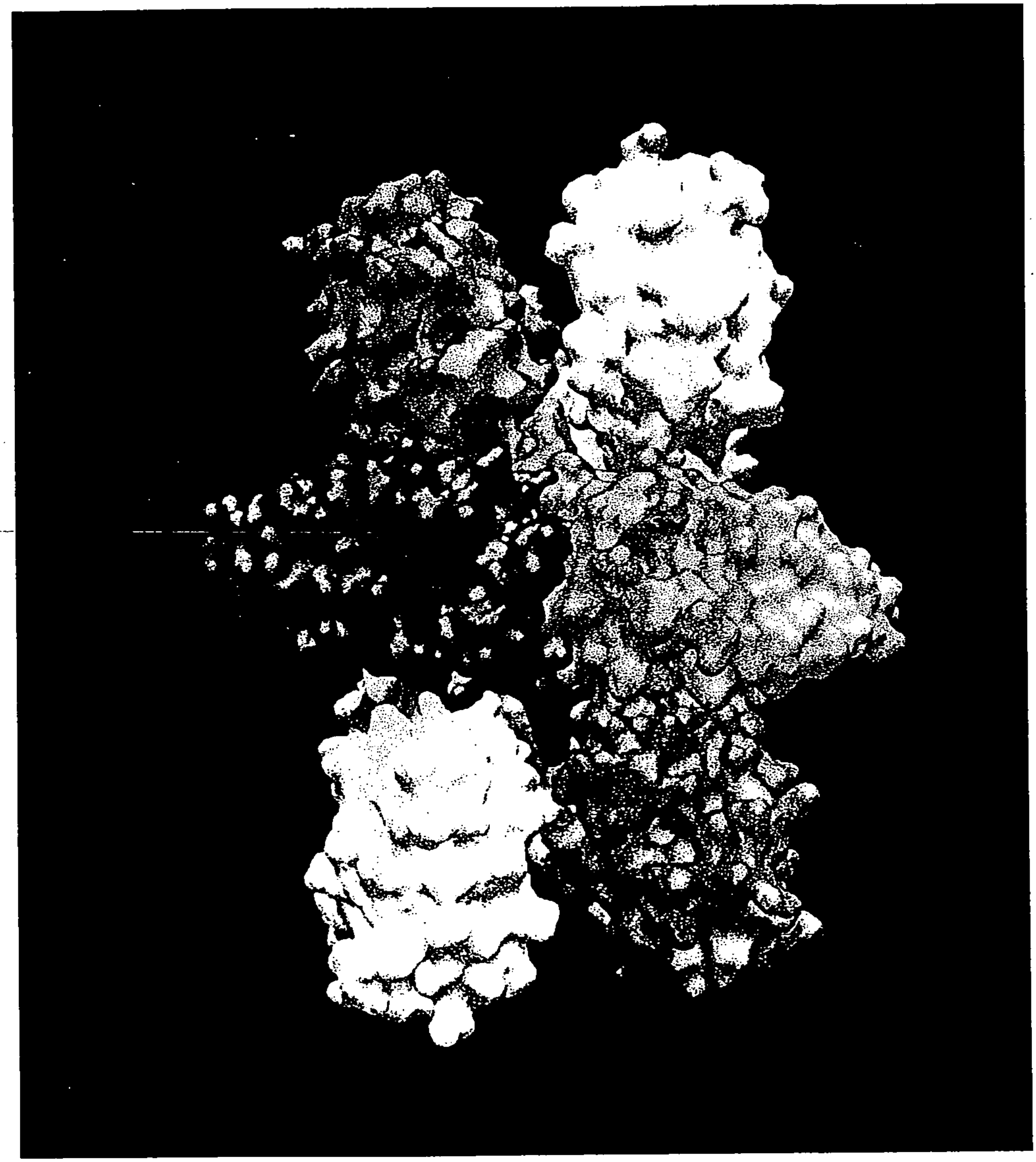
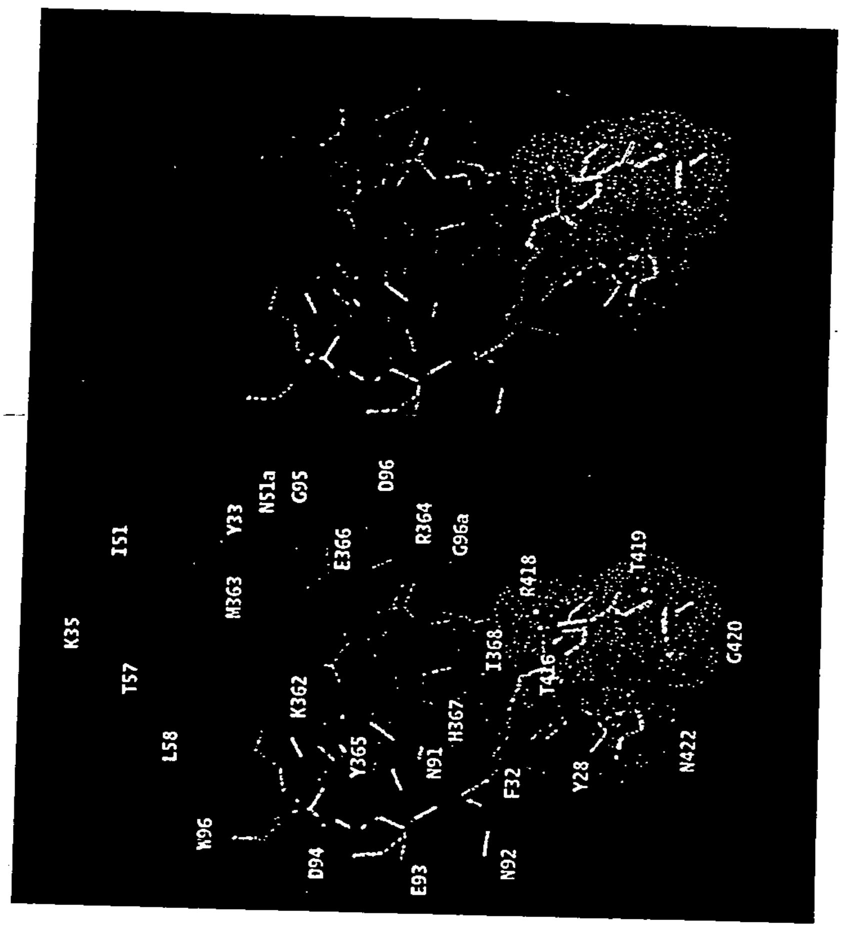


FIGURE 4


K35
I51
T57
L58
W96
M363
Y33
N51a
G95
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D96
R364
G96a
K362
Y365
H367
N91
I368
R418
T419
T416
G420
F32
Y28
N422
D94
E93
N92

FIGURE 5A

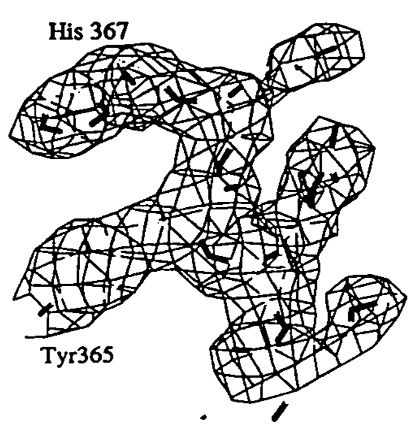
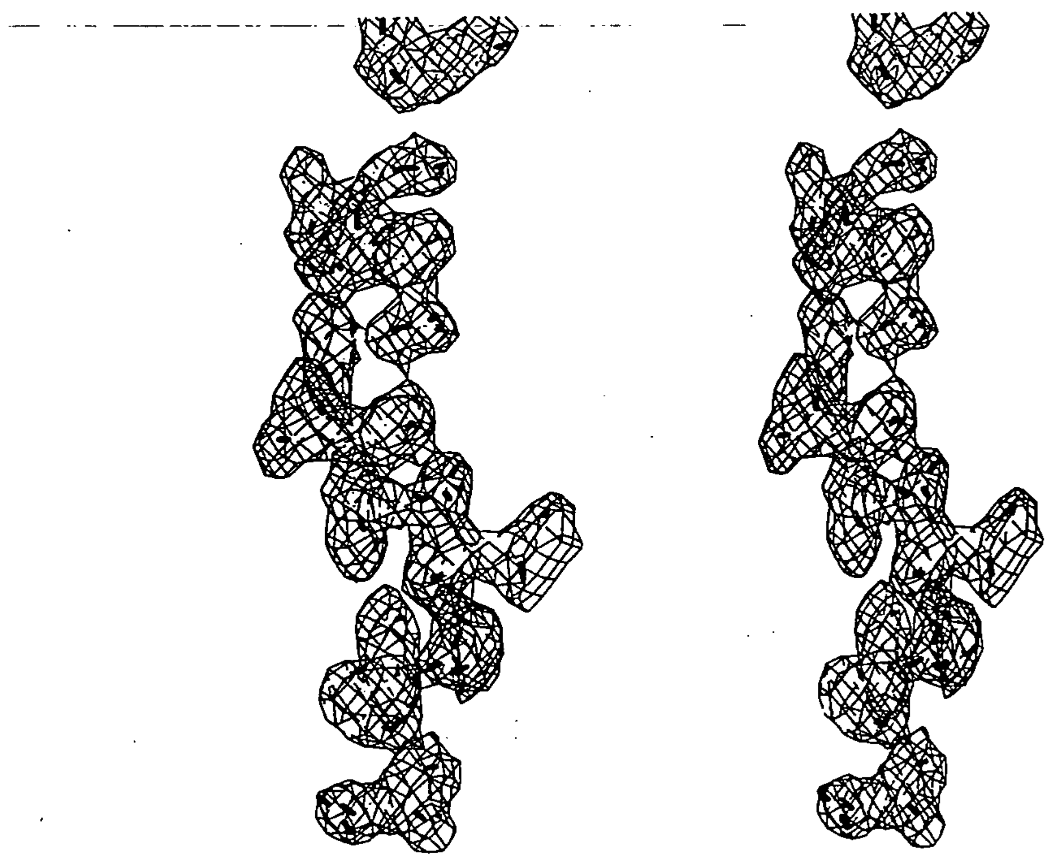
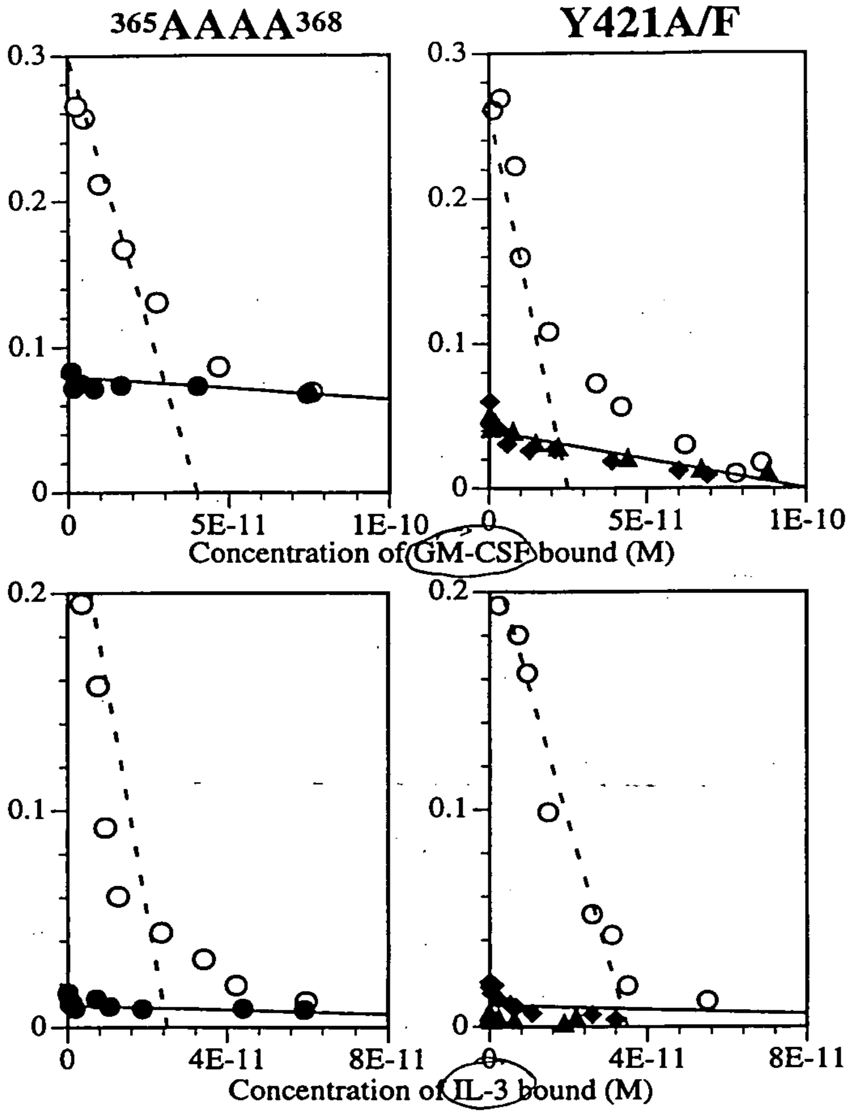


FIGURE 5B



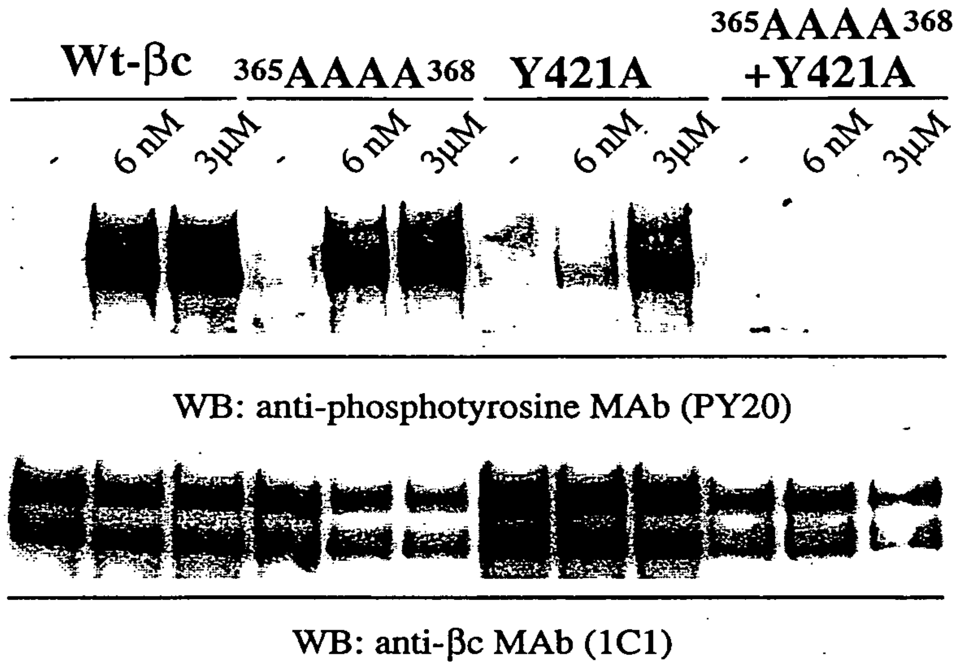
A.



365AAAA368
Y421A/F
0.3
0.2
0.1
0
0
5E-11
1E-10
Concentration of GM-CSF bound (M)
0.2
0.1
4E-11
8E-11
Concentration of IL-3 bound (M)

FIGURE 6A

B.



Wt-βc
365AAAA368
Y421A
365AAAA368 +Y421A
-
6 nM
3μM
WB: anti-phosphotyrosine MAb (PY20)
WB: anti-βc MAb (1C1)

FIGURE 6B